

THE EFFECTS OF EMBRYOS OF DIFFERENT DEVELOPMENTAL STAGES ON REPRODUCTIVE BEHAVIOR AND PHYSIOLOGY IN BROODING FEMALES OF THE AMPHIPOD CRUSTACEAN *GAMMARUS PALUSTRIS*

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ABSTRACT

The expression of male reproductive behavior is correlated with female intermolt stage in the amphipod crustacean *Gammarus palustris*. Previous studies suggested that three factors that vary with female intermolt stage influence males: (1) water-borne pheromones, (2) contact pheromones on females' exoskeleton; and (3) female behavior. The present study was conducted to determine whether a fourth factor, developmental stage of brooded embryos, might also affect male behavior. In addition, the effects of the embryos on the females' own behaviors and physiologies were examined.

The contents of the females' brood pouches were altered by removing the broods of females of specific intermolt stages, and presenting the females with embryos of other females. *G. palustris* females readily place conspecifics' embryos in their pouches. Thus males' responses to females at the same intermolt stage but with broods of different developmental stages could be observed.

The data showed that the nature of the females' brood pouches had no measurable effect on male behavior. In addition, the embryos' ages did not affect female intermolt period, reproductive behavior, or reproductive physiology.

INTRODUCTION

In some species with brood care, the presence of a brood can influence adult reproductive behavior and physiology. This has been best documented in the vertebrates and the social insects. For example, the presence of embryos in the nest of the stickleback, *Gasterosteus aculeatus*, inhibits male courtship behavior (Sevenster-Bol, 1962), and the removal of embryos from a nest of the great tit, *Parus major*, can induce courtship behavior and the production of another brood (Lack, 1966). Further, the presence or absence of eggs clearly influences egg production in at least several social insects (Wilson, 1971).

The crustaceans typically exhibit brood care, but the relationship between the embryos and reproductive behavior and physiology has not been examined in most marine species. An exception to this is the observation that the removal of the embryos from the brood pouches of female *Gammarus lawrencianus* (an amphipod) stimulates some aspects of the males' reproductive behavior (Dunham, 1986). This is an interesting discovery because the typical *Gammarus* life-history pattern (described below) suggests both the feasibility and the advantage of this potential method of regulating reproductive effort.

Gammarus females typically produce several broods in succession during the

warmer months in the temperate zone. Generally, amplexus (precopulation) begins towards the end of the female's intermolt period and continues until the female molts, at which time copulation occurs. Then, within an hour, ovulation occurs, and the male and female separate. All the embryos of the current brood are deposited in the female's brood pouch simultaneously at ovulation. Development occurs in the pouch, and the juveniles hatch and emerge shortly before the female is due to molt again [the times of hatching and emergence are species-specific (Borowsky, 1980b)]. The female remains alone until a few days before her next molt, when amplexus is reinitiated and the cycle is repeated.

Thus, all the embryos of a specific brood are at the same stage of development, the stage of development of the embryos is correlated with the female's intermolt period, and males generally initiate amplexus when the brood is in the later stages of development. Further, males should have little difficulty in determining the nature of the brood pouch contents because the pouch is open to the environment (Borowsky, 1980a), and the embryos could stimulate male behavior either by direct contact, or by water-borne substances.

Earlier studies had shown that *Gammarus palustris* females will readily place the broods of other conspecifics in their own pouches (Borowsky, 1983). This suggested that the effects of brood pouch contents on male behavior could be examined in detail in this species by exchanging embryos between females of different intermolt stages.

It was reasoned that the presence of broods could influence reproductive behavior of both sexes in several ways. First, immature embryos might inhibit these behaviors; second, mature embryos might stimulate them; and third, both of these possibilities might be true. In addition, if the embryos had some effect, it could either be direct, in which case the mere presence of the embryos would influence behavior, or it could be indirect, first modifying the female's physiology over the course of days, and then altering both sexes' behaviors. In the first case, the presence or absence of embryos would alter the animals' behaviors within a short time, but in the second case, some time would be necessary for the physiology of the female to change.

MATERIALS AND METHODS

All animals employed in the present study were obtained by hand-picking individuals from under rocks and debris in the intertidal zone at low tide at Jamaica Bay, New York, on 2 and 4 April 1986. Animals were brought to the laboratory immediately, and couples in amplexus were placed in individual 10 cm diameter glass culture dishes in water from the collecting site (about 25 ppt). They were maintained at 20°C with a light cycle of 15:9 L:D, and supplied with *Ulva lactuca* thalli *ad libitum*.

Females in amplexus and carrying embryos in the later stages of development in their brood pouches were classed as "receptive." Previous studies had shown that the intermolt period of *G. palustris* females is about eight days at 20°C, and that females who had molted two days before rarely entered into amplexus. Therefore, females who had molted and ovulated two days before and were not in amplexus were classified as "non-receptive" in this study. Eight experiments were conducted as follows: non-receptive females were assigned at random to four treatment groups; Experiment I, females were placed on frozen seawater; Experiment II, females whose embryos were removed were presented with the embryos of other non-receptive females; Experiment III, females whose embryos were removed were not presented with any embryos; Experiment IV, females were presented with receptive females' embryos. Receptive females were randomly assigned to the other four groups: Experiment V, females were placed on frozen seawater; Experiment VI, females whose embryos were

removed were presented with the embryos of other receptive females; Experiment VII, females whose embryos were removed were not presented with any embryos; and Experiment VIII, females whose embryos were removed were presented with the embryos of non-receptive females (Tables I, II). Each experiment employed ten different pairs of animals (80 tests were conducted altogether).

Embryos were removed as follows: females were placed on a bed of frozen seawater to anesthetize them, then the embryos were aspirated from the pouch with a fine pipette inserted between the brood plates. After the embryos were removed, females were placed in a clean dish with fresh seawater and allowed to recover for one day. Depending on the experiment, the females were either presented with their own embryos, with other females' embryos, or with no embryos at all (as described above). Females presented with embryos placed them in their brood pouches during the one-day recovery period.

The responses of males to females were tested after the recovery period (24 h after treatment) as follows: ten females from each experimental treatment were tested individually by placing the female and a male simultaneously in a clean culture dish with fresh seawater. Each male was obtained by gently separating it from another female immediately before a test. Three observations were made: first, the time from the introduction of the animals to the dish and the occurrence of "grabbing" behavior (the male grasps the female's exoskeleton firmly with its claws); second, the time from grabbing to either the initiation of amplexus or the separation of the couple ("contact time"); and third, the occurrence of amplexus.

"Grabbing" was noted because this is the earliest behavior in the typical sequence of reproductive behaviors which indicates unequivocally that the male is aware of the presence of the female, and "contact time" was measured because it represents the time during which the male obtains the information from the female which determines whether he will initiate amplexus (Borowsky and Borowsky, 1985, 1987). Amplexus is stereotyped and is easily distinguished from other behaviors (Borowsky, 1984).

After the behavioral observations, the couples were maintained in individual dishes until the female either molted or died. This was done for two reasons; first, to verify the viability of removed and replaced embryos by making sure they developed and hatched; and second, to determine whether the presence of embryos whose developmental stage was inappropriate for the female's intermolt period influenced behavior later in the female's cycle. Accordingly, after each couple's behavior was observed, the animals were observed daily, noting the day that the brood hatched, the day when amplexus was initiated, and the day of the female's molt.

RESULTS

There was a significant difference among the eight experiments in the number of times that amplexus was initiated ($\chi^2_7 = 52.4$, $P < 0.001$). The difference was due to female class ("receptive" versus "non-receptive"), rather than to the nature of the brood pouch contents. There was a significant difference between all non-receptive females grouped together and all receptive females grouped together regardless of brood pouch contents (Tables I and II, respectively; one of 40 non-receptive females entered into amplexus, while 32 of 40 receptive females entered into amplexus; $\chi^2_1 = 49.6$, $P < 0.001$). In contrast, there was no significant difference between females with broods and those without (33 of 60 females with embryos engaged in amplexus, and 10 of 20 females without embryos engaged in amplexus; $\chi^2_1 = 0.15$, $P > 0.05$). It is especially noteworthy that there was no significant difference among the four exper-

TABLE I

Male reproductive behaviors expressed to non-receptive females whose brood pouches had different contents

Experiments	Time to grab (s)		Time to decision (s)		Decisions Number of amplexus (of 20 tests)
	$\bar{x} \pm SD$	Range	$\bar{x} \pm SD$	Range	
I. Ice-treated: embryos not removed	122.7 \pm 127	4-446	11.5 \pm 12	1-39	0
II. Embryos exchanged between non receptive females	155.7 \pm 142	31-516	14.7 \pm 19	0-59	0
III. Embryos removed permanently	176.0 \pm 175	2-442	33.3 \pm 56	0-164	0
IV. Embryos removed and replaced with receptive females' embryos	86.3 \pm 92	0-285	33.1 \pm 41	2-147	1

iments performed on each class of female (non-receptive and receptive females, Tables I and II, respectively: $\chi^2_s = 3.08$ and 3.50 , respectively; $P_s > 0.05$). Thus, the nature of the contents of the females' brood pouches did not affect the frequency of amplexus.

In addition, the lengths of time from the introduction of pairs of animals into observation dishes to the times when males grabbed females was not significantly different among the eight experiments (Tables I and II: one-way ANOVA; $F^7_{68} = 1.55$, $P > 0.05$). Further, there was no significant difference among the groups in the lengths of time from grabbing to either amplexus or separation (one-way ANOVA; $F^7_{72} = 1.76$, $P > 0.05$). Thus, while the decision to engage in amplexus depended largely upon whether the female was receptive, the length of time employed to arrive at the decision was not.

Another effect of the brood on reproductive behavior could be to modify the female's physiology. The frequency of amplexus increases as female intermolt period advances, but there is variability among individual females' initial day of amplexus (Borowsky and Borowsky, 1985, 1987). It was hypothesized that the absence of a brood might accelerate the time to amplexus, thus enhancing the successful fertilization of the next brood. However, there was no significant difference in the time from the female's molt to the onset of amplexus between non-receptive females whose broods had been removed permanently and non-receptive females who retained their

TABLE II

Male reproductive behavior expressed to receptive females whose brood pouches had different contents

Experiments	Time to grab (s)		Time to decision (s)		Decisions Number of amplexus (of 20 tests)
	$\bar{x} \pm SD$	Range	$\bar{x} \pm SD$	Range	
V. Ice-treated: embryos not removed	26.5 \pm 22	4-79	59.2 \pm 44	16-180	7
VI. Embryos exchanged between receptive females	148.1 \pm 164	9-553	41.0 \pm 38	14-147	8
VII. Embryos removed permanently	55.7 \pm 44	3-126	41.1 \pm 16	15-76	10
VIII. Embryos removed and replaced with non-receptive females' embryos	103.9 \pm 112	4-329	31.5 \pm 23	2-74	7

broods (Experiment III vs. Experiments I and II combined, respectively; $n_s = 10$ and 20 , ranges $8-18$ and $9-22$, and $\bar{x} = 12.8 \pm 4$ and 13.6 ± 4 , respectively; Student's t -test: $t_{28} = -0.4625$).

Another way the absence of a brood might influence female physiology could be to accelerate the time until the female's next molt. However, the intermolt periods of females without broods were about the same as the intermolt periods of females with broods (Experiment III vs. Experiments I and II combined, respectively; $n_s = 10$ and 17 , ranges $12-23$ and $12-25$ days, and $\bar{x}_s = 16.9 \pm 4$ and 16.9 ± 4 , respectively).

DISCUSSION

The data show that on the basis of the criteria employed in the present study, the nature of the contents of females' brood pouches has no effect on reproductive behavior or reproductive physiology in *G. palustris*. In receptive females, the absence of broods did not increase, and the presence of immature broods did not decrease the frequency of amplexus. In the reciprocal experiments on non-receptive females, the presence of a mature brood did not increase the frequency of amplexus. Finally, the absence of a brood did not significantly hasten the onset of amplexus in non-receptive females without broods nor did it accelerate the time to these females' next molt.

These data are consistent with earlier observations of *G. palustris* reproductive behaviors which suggest that the females' behaviors and contact pheromone(s) on their exoskeleta stimulate amplexus and copulation (Borowsky and Borowsky, 1985, 1987).

But the results differ from those of Dunham (1986) who found that *G. lawrencianus* females with empty brood pouches engaged in amplexus more often than females carrying broods, even when they were at a comparable intermolt stage. There are several explanations for the differences. First, two different species were studied. Significant differences in behavior have been found between other *Gammarus* species (Borowsky, 1980b). But second, it is possible that the slightly different criteria employed for scoring amplexus accounts for the difference in results. Occasionally a male *G. palustris* carried a female in the amplexus position immediately after an encounter but dropped it shortly thereafter. Since the function of amplexus is to maintain pairing until fertilization is possible, this temporary amplexus was not considered to be biologically significant and was not scored here. Dunham's observation consisted of noting whether the pair was in amplexus three minutes after the animals were introduced to each other.

It would be interesting to test the effects of broods on the reproductive behavior and physiology of the larger Crustacea. The larger species generally reproduce infrequently [for example the lobster, *Homarus americanus*, reproduces biennially, (Aiken and Waddy, 1980)], and would stand to benefit from a mechanism which compensated for the loss of a current brood.

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